

Rabbit Hemorrhagic Disease Virus Variant Recombinant VP60 Protein Induces Protective Immunogenicity

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Received: April 1, 2015
Revised: July 16, 2015
Accepted: July 20, 2015

First published online
July 22, 2015

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pISSN 1017-7825, eISSN 1738-8872

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Rabbit hemorrhagic disease virus (RHDV) is highly contagious and often causes fatal disease that affects both wild and domestic rabbits of the species *Oryctolagus cuniculus*. A highly pathogenic RHDV variant (RHDVa) has been circulating in the Korean rabbit population since 2007 and has a devastating effect on the rabbit industry in Korea. A highly pathogenic RHDVa was isolated from naturally infected rabbits, and the gene encoding the VP60 protein was cloned into a baculovirus transfer vector and expressed in insect cells. The hemagglutination titer of the Sf-9 cell lysate infected with recombinant VP60 baculovirus was 131,072 units/50 μ l and of the supernatant 4,096 units/50 μ l. Guinea pigs immunized twice intramuscularly with a trial inactivated RHDVa vaccine containing recombinant VP60 contained 2,152 hemagglutination inhibition (HI) geometric mean titers. The 8-week-old white rabbits inoculated with one vaccine dose were challenged with a lethal RHDVa 21 days later and showed 100% survival rates. The recombinant VP60 protein expressed in a baculovirus system induced high HI titers in guinea pigs and rendered complete protection, which led to the development of a novel inactivated RHDVa vaccine.

Keywords: Rabbit hemorrhagic disease virus, vaccine, recombinant protein

Introduction

Rabbit hemorrhagic disease virus (RHDV) is one of the most fatal diseases affecting the rabbit industry in several countries. RHDV belongs to the family *Caliciviridae* and genus *Lagovirus* and has not been propagated in any continuous cell lines. The RHDV genome consists of a 7.5 kb single-stranded ribonucleic acid (RNA) encoding a protein approximately 257 kDa in size [12]. The polyprotein can be separated into several mature nonstructural proteins and a 60 kDa (VP60) capsid protein *via* proteolytic processing at eight cleavage sites [21]. Based on genetic and antigenic analyses, RHDV species can be divided into at least two subtypes, original RHDV and RHDV variant (RHDVa), and nucleotide sequence similarities between these subtypes show a 14% maximum difference. Other serotypes have not yet been identified worldwide [7].

Since the time the first case of RHDV was reported in China in 1984 [9], the disease has been reported globally and is endemic in East Asia and Europe [12, 22]. RHDV is

mainly transmitted by close contact with infected rabbits and contaminated fur. Infection causes rapid blood clot formation in the heart, lungs, liver, and kidneys. The clots compromise blood circulation, resulting in heart and respiratory failure [19]. Previous studies in Korea identified the original RHDV in 2006 and RHDVa isolates in 2007 and 2008 [15].

The inability to propagate this virus in continuous cell lines has necessitated alternative methods of antigen production for vaccine development. In the first generation, a tissue-inactivated vaccine was manufactured from the liver of rabbits infected with RHDV. Even though the tissue vaccine was efficacious, its production involved significant biological risks and posed animal welfare issues. Studies have reported use of the VP60 protein in a subunit vaccine against RHDV [9, 17]. Several antigen producing systems, such as bacteria, yeast, poxvirus-based vectors, and insect cells with a baculovirus system, have been used to generate recombinant VP60; each recombinant VP60 protein produced in these systems induced a protective humoral response

Table 1. Oligonucleotide primers used to amplify the VP60 gene of RHDVa.

Primer	Oligonucleotide sequences (5'-3')	Genomic region
VP60F	<u>CCC TCG AGT ATG</u> GAG GGC AAA GCC GCT AC	VP60
VP60R	CC <u>GAATTC</u> TGA CAT AAG AAA AGC CAT TGG TTG TGC C	
Bac F	TTT ACT GTT TTC GTA ACA ACA GTT TTG	Baculovirus
Bac R	CAA CAA CGC ACA GAA TCT AGC	Multicloning site

Underlined sequences show restriction enzyme sites (*Xho*I and *Eco*R1) and start codon.

against an RHDV challenge [1, 2, 5, 6, 8].

In a previous study, the RHDVa strain isolated in Korea in 2008 was characterized molecularly, and its pathogenicity was evaluated in animals [22]. In this study, we expressed the gene encoding the structural capsid protein VP60 using a Korean strain, KV0801, in a baculovirus system. In addition, the hemagglutinating activity of the recombinant VP60 protein was investigated in human erythrocytes. The immunogenicity of the inactivated RHDVa vaccine was evaluated in guinea pigs, and its efficacy was determined in rabbits.

Materials and Methods

Construction of a Plasmid Carrying the RHDVa VP60 Gene

Genomic RNA was extracted from the RHDVa KV0801 strain isolated from naturally infected rabbits in 2008 [21]. Each forward and reverse primer contained *Xho*I and *Eco*R1 restriction enzyme sites, respectively (Table 1). The primers were designed based on the genomic sequence of recent RHDVa isolates of KV0801 (GenBank Accession No. FJ212322). The RHDVa VP60 gene was amplified by reverse-transcription polymerase chain reaction (RT-PCR) using the specified primer set, and the products were separated on a 1.8% agarose gel. After purification, the PCR products were ligated into the cloning site of the pGEM-T vector system (Promega, USA). The VP60 gene fragment was obtained using *Xho*I/*Eco*RI double digestion of a pGEM/VP60 plasmid and ligated into the *Xho*I/*Eco*RI site of the baculovirus transfer vector, pBlueBac4.5/V5-His (Invitrogen, USA), which contains a C-terminal peptide encoding a 6-histidine tag for detection and purification. The plasmid expressing the VP60 gene was transformed into DH5 α competent cells. The pBlueVP60 plasmid was extracted and purified using a plasmid purification kit (Qiagen, USA).

Construction of Recombinant VP60 Baculovirus

To construct the recombinant VP60 baculovirus, Bac-N-Blue DNA (Invitrogen, USA) and 10 μ g/ μ l purified pBlueVP60 plasmid DNA were mixed with cellfectin reagent (Invitrogen, USA) in Grace's insect medium without supplements. After incubation for 15 min at room temperature, the transfer mixture was added to a 60 mm dish of *Spodoptera frugiperda* (Sf-9) cells cultivated at 27°C. After 3 days, the supernatant was harvested, and the cells were

incubated continuously by adding fresh medium containing fetal bovine serum (FBS). A plaque assay was performed to purify the recombinant baculovirus in 1% agarose medium containing 150 μ g/ml X-gal. A PCR assay targeting the recombinant VP60 baculovirus using baculovirus-specific primers confirmed the isolation of a pure plaque (Table 1). The recombinant VP60 baculovirus was passaged three times by infecting Sf-9 cells at a MOI (multiplicity of infection) of 0.1. The third passage was used as the viral stock and propagated for the production of recombinant VP60 protein in Sf-9 cells.

Preparation of an Experimental Vaccine

The recombinant VP60 baculovirus was used to prepare the experimental vaccine *via* inoculation into Sf-9 cells cultured in Grace's medium supplemented with 10% FBS, penicillin (100 IU/ml), streptomycin (10 μ g/ml), and amphotericin B (0.25 μ g/ml). The Sf-9 cells displaying cytopathic effects were harvested by centrifugation at 1,500 \times g for 5 min and resuspended in phosphate buffered saline (PBS, pH 7.2) at 10% of the original volume. The suspended solution underwent three freeze-thaw cycles and was centrifuged at 3,000 \times g for 30 min to remove cell debris. The harvested recombinant VP60 proteins were adjusted with PBS to 10,000 HA units/50 μ l and inactivated using 0.2% formaldehyde at 37°C for 24 h. After inactivating the baculovirus, the antigen and rehydragel as adjuvant (Seppic, France) were mixed in a 9:1 ratio under agitation.

Immunofluorescence Assay

To identify the recombinant VP60 protein, Sf-9 cells were infected with recombinant VP60 baculovirus in a 96-well microplate and incubated for 4 days. The infected cells were fixed using cold acetone at -20°C for 15 min, washed with PBS (pH 7.2), and then incubated with a RHDV polyclonal antibody (Animal and Plant Quarantine Agency; QIA, Korea) and 6-histidine monoclonal antibodies (Sigma) for 1 h at 37°C. After washing the plate, anti-

Table 2. Hemagglutination assay activity of recombinant VP60 protein.

Designation	HA titer	Red blood cell
Supernatant	4,096	Human type "O"
Cell lysate	131,072	

rabbit and anti-mouse IgG fluorescein isothiocyanate (FITC) conjugates were added and incubated under the same conditions. After rinsing with PBS, the cells were examined using a fluorescence microscope at 400× (Nikon, Japan).

Hemagglutination Assay (HA) and Hemagglutination Inhibition (HI) Test

For the HA, 50 µl of recombinant VP60 protein was added to the first row of a 96-well microplate, and a serial 2-fold dilution was performed using PBS (pH 7.2) containing 3% bovine serum albumin. The same volume of O-type human erythrocytes (0.6%) was added and then incubated for 1 h at 4°C. The HA titer was expressed as the reciprocal of the highest dilution showing agglutination. The HI test was performed according to the OIE manual in 96-well round bottom microplates [15]. Briefly, to remove any nonspecific inhibitors, 50 µl of serum was mixed with 450 µl of 25% kaolin and incubated for 30 min at room temperature. After incubation, the kaolin was removed by centrifugation at 3,000 ×g for 15 min in a microfuge. The clear supernatant was mixed with 25 µl of packed human O-type erythrocytes to remove any natural agglutinins. After incubation for 1 h at 37°C, the treated serum was separated from the erythrocytes by centrifugation. Then, 4–8 HA units of RHDV (RHF89 strain) was added to 25 µl of treated serum. After incubation for 1 h at room temperature, 50 µl of 0.6% human O-type erythrocytes was added to the serum, and the microplates were incubated at 37°C for 1 h. The HI antibody titer was expressed as the reciprocal of the highest serum dilution showing complete inhibition of hemagglutination.

Animal Experiment

The experimental design on the basis of Korean Standard Assay of Veterinary Biological Products was submitted to the laboratory animal ethics committee (QIA, Korea) and was approved by the

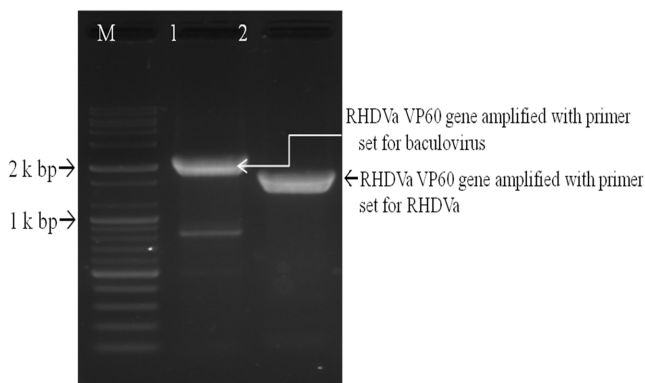


Fig. 1. Identification of the RHDVa VP60 gene from cloned RHDVa VP60 baculovirus.

Lane M: 1 kb DNA ladder; lane 1: RHDVa VP60 gene amplified with specific primer set for baculovirus; lane 2: recombinant RHDVa VP60 baculovirus.

committee (NVRQS AEC 2010-44). Eight 7-week-old guinea pigs with no antibodies against RHDV were immunized intramuscularly with 1 ml of the inactivated RHDVa vaccine, containing over 10,000 HA units, twice at 2-week intervals. Four guinea pigs in the control group did not receive any treatment. Six rabbits were inoculated with one dose of vaccine once, and two rabbits served as controls. All rabbits were challenged intramuscularly with 100 50% median lethal dose (MLD₅₀) units (16 HA units) of a virulent RHDVa strain, KV0801, 21 days after vaccine inoculation. The rabbits were observed daily, and the survival rate was determined 14 days following the challenge. Blood samples were collected from immunized guinea pigs to determine the immunogenicity and from rabbits to evaluate the efficacy of the vaccine.

Statistical Analysis

A one-way ANOVA test was performed by GraphPad Prism (ver. 6.05) for statistical analysis. $p < 0.05$ was considered to indicate statistical significance.

Results

Expression of Recombinant VP60 Protein in Insect Cells

The RHDVa gene, encoding VP60, from the KV0801 strain was amplified and cloned into pGEM-T and pBluBac4.5/V5-His vectors, with a 6-histidine tag in the C-terminal region. After its cleavage by restriction enzymes from the transfer vector, the size of the VP60 gene was 1,740 bp (Fig. 1). The plaque-purified recombinant VP60 baculovirus was detected by both anti-guinea pig RHDV polyclonal antibodies and 6-histidine monoclonal antibodies, using an indirect fluorescence assay (Fig. 2). The recombinant VP60 baculovirus was propagated in Sf-9 cells, and the VP60 protein was measured using the HA. The HA titer was 131,072 and 4,096 units/50 µl in the cell lysate and supernatant, respectively.

Immune Response in Guinea Pigs Inoculated with the Inactivated RHDVa Vaccine

Guinea pigs were immunized intramuscularly twice with inactivated vaccines containing recombinant VP60 protein. The HI antibody titers are shown in Fig. 4A. The post-inoculation HI titer was higher at 28 days than at 14 days. Subjects in the control group did not have a detectable HI antibody response.

Survival Rate of Immunized Rabbits against Lethal RHDVa

To determine if the recombinant VP60 protein expressed in insect cells was biologically functional, 8-week-old white rabbits were inoculated with one dose of the vaccine and were challenged 21 days later with a lethal RHDVa strain.

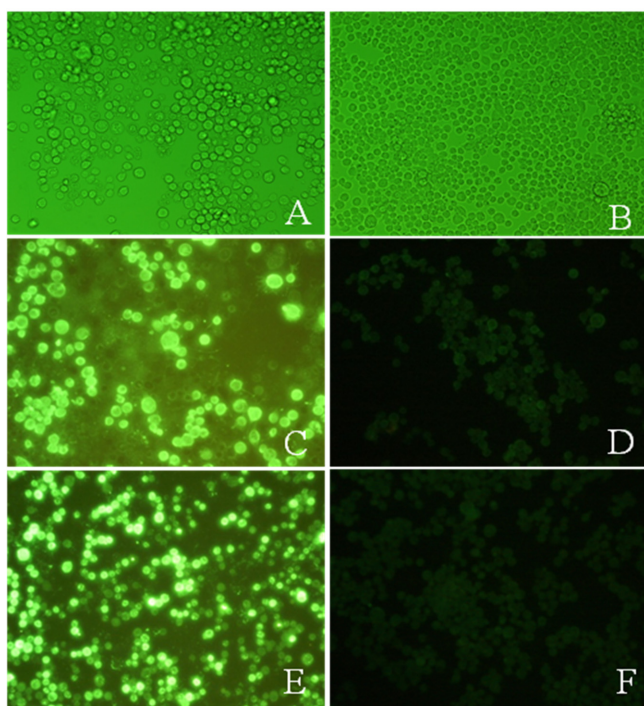


Fig. 2. Cytopathic effect in Sf-9 cells infected with recombinant RHDVa VP60 baculovirus (A, $\times 200$) and normal cells (B, $\times 200$). Identification of recombinant RHDVa VP60 baculovirus by immunofluorescence assay in Sf-9 cells using anti-guinea pig RHDV polyclonal antibody (C, $\times 200$) and 6-histidine monoclonal antibodies (E, $\times 100$). Normal Sf-9 cells were used as control cells (D, $\times 200$; F, $\times 200$).

As Fig. 3 showed, the immunized rabbits showed 100% survival rates without any clinical signs of rabbit hemorrhagic disease (Korean patent number: 10-1258633). There were no surviving rabbits in the control group by 14 days. HI titers before the challenge ranged from 1:40 to 1:80 in rabbits immunized with the vaccine, and increased to 1:320 to 1:2560 after the lethal challenge. No detectable HI antibody response was observed in the control group at the time of challenge (Fig. 4B).

Discussion

Since 1984 when RHDV was first identified in rabbits, there has been a need for an effective vaccine to reduce damages suffered by the rabbit industry. The lack of viral propagation in continuous cell lines has restricted the development of new vaccines. The first generation of vaccines, a tissue-inactivated vaccine, poses a biological risk and is controversial owing to animal welfare issues.

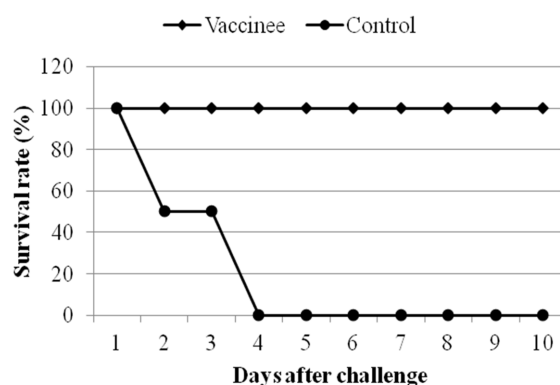


Fig. 3. Survival rate in rabbits immunized with the inactivated VP60 vaccine *via* the intramuscular route and challenged with the highly pathogenic RHDVa strain (KV0801 strain).

The major RHDV structural protein, VP60, used in second-generation vaccines, has been produced using a number of expression systems, such as *E. coli*, insect cells, yeast, and mammalian cells [1, 2, 5, 8, 11]. The recombinant VP60 protein was shown to induce protection against lethal RHDV challenge in rabbits [18].

In this study, the VP60 gene from the KV0801 strain, a RHDVa, was cloned and expressed in a recombinant baculovirus as a potential vaccine and a diagnostic reagent for the HI test. Baculovirus expression systems have advantages such as high expression, eukaryotic post-translational modifications, and self-assembly of the viral capsid protein into virus-like particles (VLPs) [3]. The Sf-9 cells infected with recombinant VP60 baculovirus were harvested and examined using the HA. Our results showed that the recombinant VP60 protein expressed in a baculovirus system was produced at high levels (4,096 unit/50 μ l), assuming that the recombinant VP60 protein is expressed as releasing form like VLPs. The HA antigen was obtained from the homogenized liver of a rabbit inoculated with wild-type RDHV and used to perform HI. It was reported that the recombinant capsid protein expressed in baculovirus was morphologically and antigenically indistinguishable from the native virus [14].

Several studies reported that the serological immune response plays a key role in protection, and the cellular immune response may also play a role in clearing the virus and preventing RHDV infection [4, 20]. In this study, eight guinea pigs inoculated with the inactivated RHDVa vaccine had high HI titers against RHDV, ranging from 1:640 to 1:20,480, indicating that this vaccine successfully induces immunogenicity in guinea pigs. Additionally, six

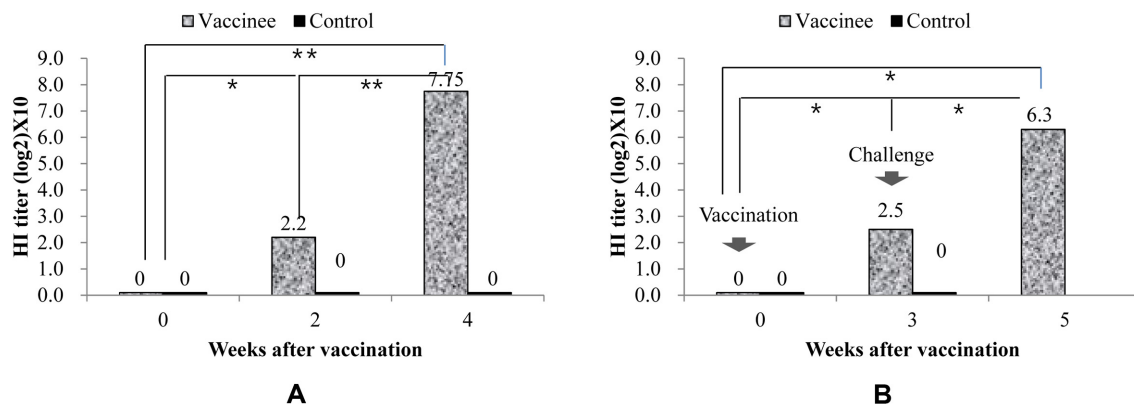


Fig. 4. Geometric mean HI titer of guinea pigs ($n = 8$) (A) inoculated with the inactivated RHDVa vaccine containing recombinant VP60 protein and rabbits ($n = 6$) (B).

* $p < 0.05$ (one-way analysis of variance).

seronegative adult rabbits inoculated with one dose of the inactivated vaccine survived a challenge with 100 MLD₅₀ units. These results suggest that the recombinant RHDVa VP60 induces protective immunity against lethal RHDVa.

In conclusion, the VP60 gene from RHDVa was cloned and expressed in a baculovirus system. These results demonstrated that the recombinant VP60 protein was able to elicit a significant humoral immune response in guinea pigs and rabbits. This is a promising vaccine for protection against RHDVa infection. However, further studies are required to examine the cellular immune response, which is closely involved in clearing the virus and preventing RHDV infection. Further clinical studies will need to be conducted in the field trial.

Acknowledgments

This work was supported financially by a grant from the Animal and Plant Quarantine Agency (B-AD14-2012-13-01), Ministry for Food, Agriculture and Rural Affairs (MAFRA), Republic of Korea.

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